Odette ROHFRITSCH

Institut de Biologie moléculaires des Plantes, C.N.R.S., Strasbourg

MORPHOLOGICAL AND BEHAVIOURAL ADAPTATIONS OF THE GALL MIDGE *LASIOPTERA ARUNDINIS* (SCHINER) (DIPTERA, CECIDOMYIIDAE) TO COLLECT AND TRANSPORT CONIDIA OF ITS FUNGAL SYMBIONT

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The gall midge *Lasioptera arundinis* attacks lateral shoots of the stems of the reed (*Phragmites australis* Trin.) with the help of its fungal symbiont (of the genus *Macrophoma).* Together with the fungus, the larvae move up the shoot and penetrate into the stem. Thus allowing both organisms access to vascular tissues of the host. The larvae feed on the host tissue and on the fungus and the overwintering larvae are not harmed by the fungal mycelium. This narrow relationship between a gall midge and fungus is an example of obligate mutualism. The female imago and the first-larval instar have evolved specific behavioral traits and stuctural adaptations to disseminate the fungus. The fungal conidia are collected on upper internodes of galled shoots by the female just before oviposition. The female transports the conidia to an oviposition site in specialized structures on the cercus (tip of the ovipositor). These structures, called mycangia, are composed of a sclerotized plate covered with large spoon-like spines which collect the conidia of the right size and shape. The conidia slide down the collecting spines and enter laterally situated pouches beneath the spines. The mycangia join the extremity of the oviduct such that eggs and conidia are deposited together. The first larval stage with its long bristles and numerous spines carries the fungal material and disseminates it along the feeding route.

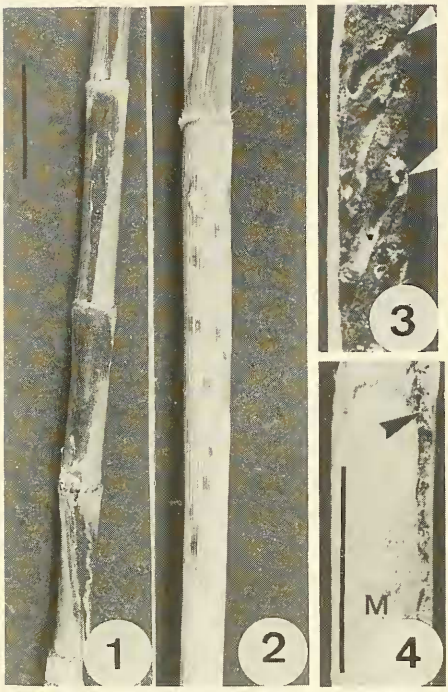
O. Rohfritsch, I. B. M. P., 12, rue du Général Zimmer, 67084 Strasbourg cedex, France.

Key words. – *Lasioptera arundinis*, *Phragmites australis*, fungus, mycangia, gall, symbiosis.

There are approximately 4300 described species within the family Cecidomyiidae (Diptera: Nematocera); about half are gall inducers (Felt 1940) and the others are detritus feeders. The ancestral feeding biology of gall midges was thought to be mycetophagy (Mamaev 1968, Gagné 1986, Roskam 1992) whereas the derived taxa belonging to the tribes Cecidomyiinae and Oligotrophini induce galls, as do most of the Lasiopterini and all of the Asphondyliini. Many of the Asphondyliini and Lasiopterini exhibit a peculiar feature that is reminiscent of the ancestral mycetophagous condition -the surface of their gall chamber is covered with a layer of fungal hyphae on which the larvae feed. These are ambrosia gall midges which are analogous to ambrosia beetles (Neger 1913) that also feed on symbiotic fungi.

Gall midges of the genus *Lasioptera* which often occur on stems of reeds and grasses (Gagné 1989) are usually associated with a fungal symbiont. Meyer (1952) observed that the gall of *L.rubi* (Schrank) on *Rubus caesius* L. lacked the characteristic layer of nutritive cells found in most galls, and instead a mycellium was present along the surface of the larval chamber with intracellular haustories within tissues of the gall. In contrast, the larvae of some *Lasioptera*, such as *L. berlesiana* Paoli, stay in preformed galleries and feed on a fungal associate that grows in tunnels which begin as tephritid oviposition holes on olives (Solinas 1967). *L. donacis* (Coutin and Faivre-Amiot 1981) feeds on the fungus *Aspergillus niger* V. Thieg. growing in old galleries of a chloropid fly in leaf sheaths of *Arundo donax* L.(Coutin and Faivre-Amiot 1981). Hermann et al. (1993) described the association of *L. ephedricola* Cockerell with the fungus *Aureobasidium pullulans* (de Bary) Arnaud which forms a dark ring around gall chambers on *Ephedra trifurca* Torr.

Skuhravá and Skuhravý (1981, 1992) described the life cycle of *L. arundinis* Shinner and *L. hungarica*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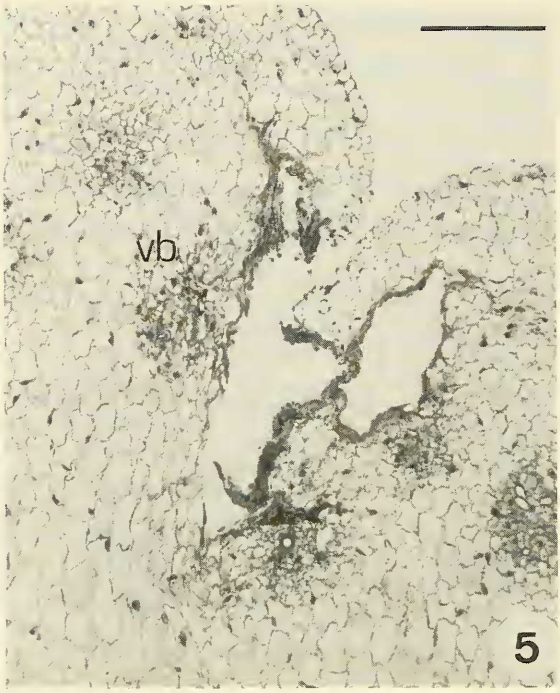
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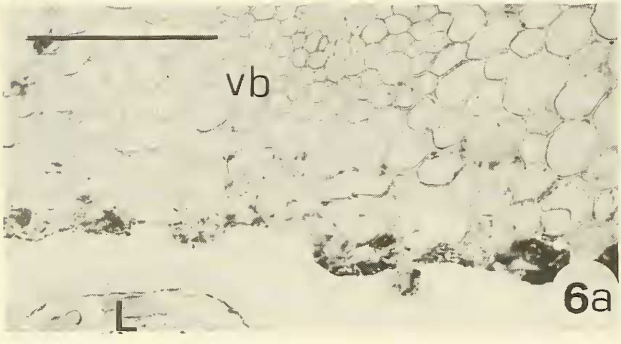
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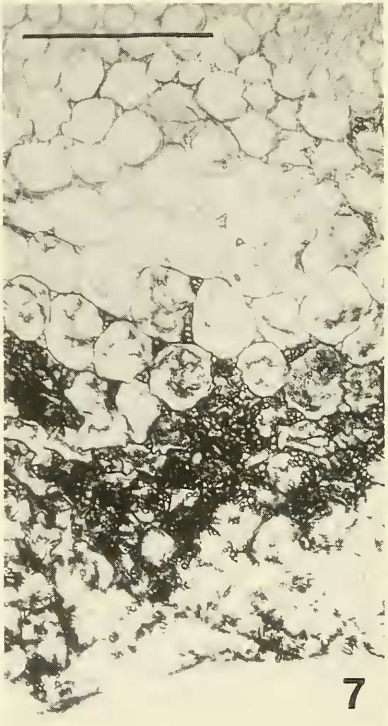
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Möhn which induce galls within stems of the common reed *Phragmites australis* Trin. in central Europe and found that galls of both contain a fungus considered to be of the genus *Sporothrix*. Developmental morphology of the gall of *L.arundinis* has also been described (Rohfritsch 1992) and at the end of this study, it was suggested that the fungus has a narrow and obligatory relationship with the inducer. It was suggested that without the larvae, the fungus would be unable to attack the stem of reeds and that the larvae need the fungus to penetrate to the stem medulla and to obtain food. It is known that the first-instar larvae of *L.arundinis* carry the conidia upwards within the stem and that all nearby larvae follow the same entrance route. However, the question remains as to how the first-instar larvae come in contact with the fungus.

There have been several suggestions as to the mechanism by which fungi are brought into the galls including non-specific air borne inoculation (Batra and Lichtwardt 1963), contamination of the eggs by adult feces (Haridass 1987), and the specific transfer of fungal spores by the ovipositor or abdominal mycangia (Bissett and Borkent 1985). Mycangia are pockets on the bodies of insects that selectively collect and transport fungi (Batra and Lichtwardt 1963). According to Borkent and Bissett (1985), the conidia carried by Lasiopterini are entrapped by two dorso-lateral groups of strong setae on uromerVIII. These authors also observed conidia among the setae of the cercus; however, in another study, Tastás-Duque and Sylvén (1989) were not able to find conidia on the setae of uromerVIII of *L. rubi*. Hermann et al.(1993) found that adults of *L. ephedricola* have structures on their ovipositors which could serve as mycangia, but were unable to find fungal propagules on the ovipositors of newly emerged females. Hermann et al. (1993) concluded that host leaves were the source of fungi for either newly hatched larvae, or females prior to oviposition, or that oviposition occurs on or near an inoculum source.

The purpose of the present paper is to demonstrate that: 1) females emerging from their pupae do not carry the fungus, 2) females collect conidia on the upper internodes of attacked shoots, 3) mycangia are present on the cercus which selectively collect conidia from the epidermis of the leaf sheath, 4) conidia and eggs are deposited simultaneously, 5) larvae disseminate the fungus along the dispersal route and aid its progress into the stem.

Material and methods

Insect rearing

Lateral shoots of common reed attacked by *L. arundinis* were collected in January from the 'Parc de Pourtalès' near Strasbourg, France. The whole shoots, about 25cm long, were kept at room temperature (22°) in a glass jar covered with muslin under natural daylight.

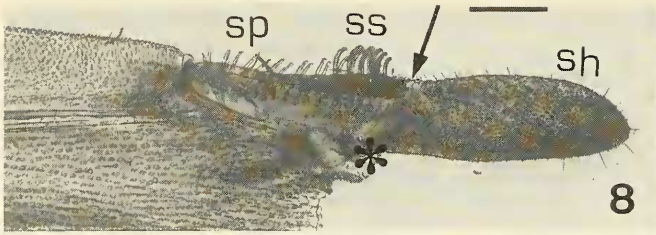
Preparation for light microscopy

Non-fixed material. – The fungal flora present on the leaf sheaths was observed in one of the staining solutions described below either separately from the plant or on a peel of the epidermis. The appendages of the ovipositor and the cercus were observed in a similar way. Stains used were: (1) coton blue which selectively stains the fungal mycelium, (2) lactic reagent (Gazet du Châtelier 1948, Rohfritsch 1992b), which makes the tissue partially transparent and stains starch, oils, cutin and lignin, (3) toluidine blue which stains nucleus, cytoplasma and cell walls.

Fixed material. – Fixative: Formaldehyde-Alcohol-Acetic acid (ethanol 70% 90 ml, glacial acetic acid 5ml, formaldehyde 5ml). Portions of the internodes (about 3cm long) were fixed over a period of 24h at room temperature. Smaller pieces of the fixed material (1cm) were embedded in paraffin and sectioned in 3-5 µm slices with a microtome (Jung, Heidelberg) and transferred to glass slides. After paraffin removal, the sections were stained with toluidine blue or with safranine-light green.

Dissections were photographed with a Zeiss Tessovar while histological sections were photographed with a Leitz microscope.

Figs. 1-7: Lateral shoots of common reed attacked by *Lasioptera arundinis* and its associated fungus. Bar scale: 0.5 cm for Figs. 1-4. 100 µm for figs. 5-7. – 1, Attacked shoot with mature larvae, basal internodes. The fungal mycelium has plugged the entrance split; 2, Upper internode of the attacked shoot. The fungus has grown through the leaf sheath and conidia are present on the epidermis; 3, Dissection of an internode filled with fungus, which contains numerous larval chambers (arrows); 4, Dissection of an upper internode. The fungus (arrow) is only present in the stem cortex, without the larva it does not reach the medulla (M). – Figs. 5-7: Cross sections of galled stems; 5, Section through the cortical tissue of a young reed stem. Both larvae and fungus have penetrated the medulla. The entrance cavity is covered by the fungal mycelium. The vascular bundles (vb) are accessilble to the feeding larva; 6, Section through a maturing gall. All stem cells are hypertrophied. The entrance split is filled with a mat of black mycelium (arrow). The fungus has attacked the most inward vascular bundles (vb). The larva is feeding on young fungal hyphae growing on the plant tissue; 6a, Detail of fig.6 showing the larval feeding site; 7, Section through a mature gall. The larva has stopped feeding, the black fungal mycelium fills the whole medullar cavity.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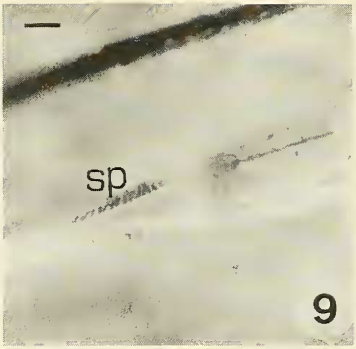
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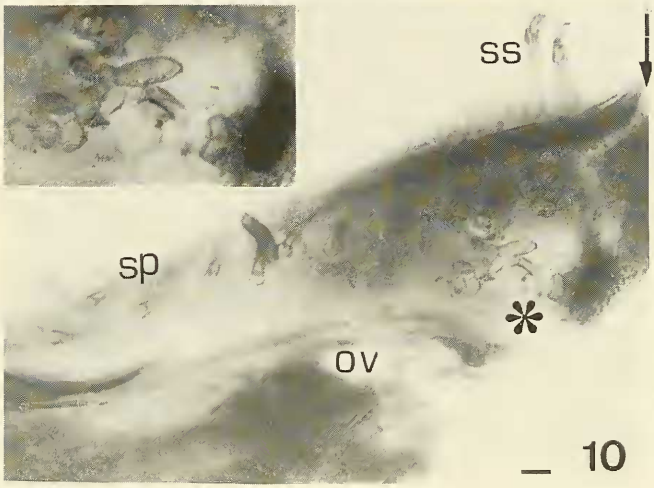
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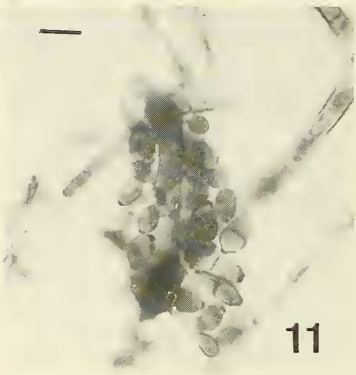
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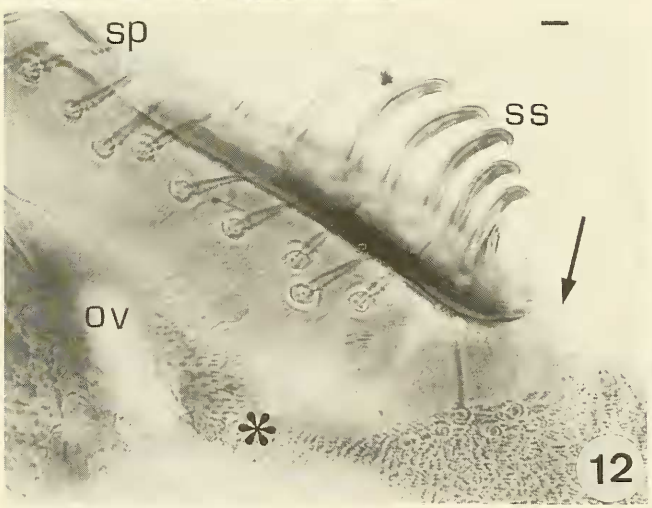
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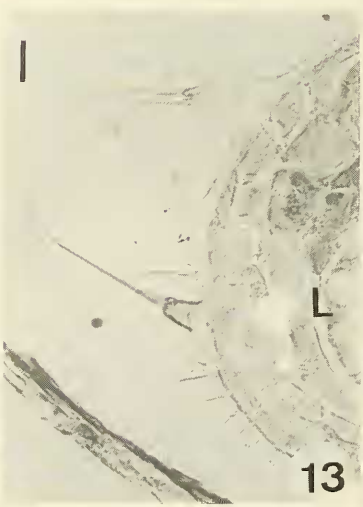
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Results and discussion

Biology of *L.arundinis* and gall development

Larvae of *L.arundinis* and its associated fungus produce a thickening and shortening of lateral shoots of common reed. Galls are made from hypertrophied stem tissue and hypertrophied medulla (figs. 1 and 3). There are 1 to 25 larvae per internode, with most larvae found in the central part of the shoot. Each internode constitutes one larval chamber in young galls. Later, the proliferating fungus isolates each larva in an individual chamber, so that the mature gall appears as multi-chambered. The uppermost internodes are free of larvae and fungi but between galled and normal tissues 1 or 2 internodes are superficially attacked by the fungus (figs. 2, 4). Overwintering larvae use their spatula to bore an exit hole in mid-May. Adults exit the gall at the end of June. Only lateral shoots damaged at their tops by other invertebrates are attacked. Each female lays from 60 to 110 eggs under the basal leaf sheath (Skuhravá & Skuhravý 1981). All freshly hatched larvae move along the same route towards the apex. The larvae carry conidia and disseminate the fungus along their route. In addition, the larvae attack the plant tissue with their mandibles, creating a stress and a discrete wounding on the plant epidermis. The fungus is now able to penetrate into the epidermis and invade the cortical tissue of the stem (Rohfritsch 1992). With the help of the fungus, the larvae enter the stem and move upwards; the fungus produces a longitudinal gallery in the cortical tissue which allows the larvae to progress through the nodes of the stem upwards. The fungus produces cell dissociation and the larval activity enlarges the cavity (fig. 5). Both organisms enhance proteosynthesis of adjoining cells, with some cells developing the cytological characteristics of nutritive cells. When the third-instar larvae reach the medulla, the fungus invades the medullar parenchyma and the innermost vascular bundles (figs.6, 6a). The mycelium soon covers the larval cavity and fills the medulla once the larvae stop feeding (figs. 3, 7) (Rohfritsch 1992).

Emergence of the gall midge and conidia collection

Attacked shoots were collected at the end of January and observed regularly each week. Within 2 weeks, the larvae bored exit holes through the fungal mycelium and the leaf sheaths, except the outermost sheath. Adults exited the galls 3 weeks later. Males emerged first and newly emerged females were free of fungal material. The ovipositor was retracted after mating, then the females flew about and finally alighted on the leaf sheath of an upper shoot internode. The ovipositor was extruded and the surface of the leaf sheath was palpated. The visited internode was not galled but the fungus was present along a narrow strip in the stem cortical tissue. From this superficially located infection, the fungus had grown through the leaf sheath and produced mycelium and conidia on the epidermis (figs. 2, 11). As was generally observed with ambrosia gall midges (Borkent & Bissett 1988), we never observed *L. arundinis* picking up fungal material directly from the gall surface. The observed *Lasioptera* gall midge found the fungal conidia on the galled host plant but not on the gall directly. It is not known how the females distinguish the 5 or more different fungi present on the surface of the same leaf sheath.

Mycangia

Borkent & Bissett (1988) reported that female *Lasioptera* species use specialized structures located on uromerVIII of the female abdomen to carry conidia and transfer them to the host plant during oviposition. Like Tastás-Duque & Sylvén (1989), I was unable to find conidia on uromer VIII, even after the female had visited the upper internodes of the galled shoot (fig. 9). However by examining the whole ovipositor in lactic reagent, I found distinct mycangia on the cercus near the tip of the ovipositor, close to the outlet of the oviduct (figs. 8, 10, 12). The paired mycangia were made up of pockets located beneath the sclerotized plate which carried large spoon-like spines. These spines have been previously observed

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Figs. 8-13: Structural adaptations of *Lasioptera arundinis* for carrying fungal symbiont. Bar scale : 50µm in figs. 8 and 9,10µm in all other figures. – 8: Posterior part of the ovipositor with the mycangial structures on uromer X. The superior lamella of uromer X is covered with spines(sp) and spoon-like sensilla (ss). The apical portion of the superior lamella is covered with sensory hairs (sh). Arrow indicates the entrance into the mycangia. Asterisk indicates the outlet which joins the oviduct. The ovipositor was processed in lactic reagent, and photographed in ethanol; 9, Portion of ovipositor showing spines (sp) on uromer VIII; 10, Mycangial pouch on uromer X. The conidia are collected by the spoon-like spines on the sclerotized plate. The conidia glide down the spines and enter the pouch (the entrance is indicated by an arrow). During oviposition, the conidia leave the pouch via the outlet (asterisk) which joins the oviduct (ov). Stain: lactic reagent. Insert: Detail of the conidia present in the mycangial pouch; 11, Conidia observed on the leaf sheath of the upper internode of the attacked shoot. This is the same internode shown in fig.2. Stain: lactic reagent; 12, Same mycangia as in fig.10, mycangia were observed in ethanol after treatment with lactic reagent, (the conidia have been removed) ss: spoon-like spines, ov: oviduct. Entrance into the mycangia is indicated by an arrow; outlet indicated by asterisk; 13, Section of first- instar larva (L) showing long bristles and numerous spines.

on other *Lasioptera* species (Skuhravá & Skuhravý 1981, Tastás-Duque & Sylvén 1989); however, the pockets just beneath the spines have not been seen. I found a few conidia on the spoon-like spines and at least 30 conidia of the same type in each pocket beneath the spines. The shape and the size of conidia corresponded with the shape of the spines. The spines evidently pick-up the conidia when the ovipositor laterally palpates the surface of the leaf sheath, retaining only conidia of the right size and shape. The conidia then slip to the base of the spine and, with the help of other spines, are guided into the cavity opening at the top of the two laterally situated pouches, beneath the sclerotized plate (fig. 10). Five females were examined and only one type of conidia was present in their mycangia. Small fragments of mycelium were sometimes present. An exit hole at the bottom of the mycangia joins the extremity of the oviduct (figs. 10, 12) such that during oviposition both eggs and conidia are deposited.

The mycangia of *L. rubi* are similar to the structures described above. Because the female could not find its fungal symbiont on the gall surface or along nearby plant surfaces, the mycangia were empty (unpublished results).

Fungus

The fungus previously associated with this gall was described as belonging to the genus *Sporothrix* of the Deuteromycetes (Skuhravá & Skuhravý 1981); however, the size and shape of the conidia are similar to those of the genus *Macrophoma* (Borkent & Bissett 1988). Borkent & Bissett suggested that the fungal genus *Macrophoma* was specifically associated with the ambrosia gall midges. The conidia carried by *L.arundinis* had a distinct terminal structure, a sort of dense rim at their top (fig. 11). Otherwise, the conidia shared the characteristic features of *Macrophoma* conidia, as described in Borkent & Bissett (1988) with similar shape, a truncated base, indicative of holoblastic ontogeny, they were first hyaline and aseptated, and at later stages developed brownish pigments and one septa. According to Borkent & Bissett these ambrosia fungi may be referred to the coelomycetes anamorphous genera and all the anamorphous observed in the gall midge mycangia could be considered congeneric.

The nutrition of the fungus was biotrophic and it was feeding inter- and intracellularly without ever killing the cells. The fungus had long slender 'prospektiv' hyphae which dispersed intercellularly, and were directed straight to the vascular bundles. These hyphae produced cell wall dissociation. Along this open route and especially near the larvae, large cytoplasma-enriched hyphae developed intercellularly with haustoria inside the cells. Near the end of larval development, the old, highly chitinized mycelium formed a dense mat along the medullar cavity and, once the larvae stopped feeding, they filled the cavity. This mycelium was unable to fruit on the gall surface. Along the entrance canal, the plant reacted by lignifying its cell walls such that the fungus was stopped in its lateral progression.

Thus the fungus can only develop towards the center of the stem. From the medulla the fungus attacked only the innermost vascular bundles and fructification did not occur. No larvae were observed along the youngest attacked internodes; the fungus invaded only the cortical parenchyma and produce a superficial gallery along the internode. From here the fungus was able to produce fructifications by growing through the leaf sheath (figs. 2,4, 11).

Fungal dispersion via the larva

First-instar larvae have unusually long bristles and many spines (fig. 13), between which fungal material was found. It is via these bristles and spines that the fungus comes in contact with the young epidermis of the host. Larval activity also results in cell wounding (Rohfritsch & Shorthouse 1982, Rohfritsch 1992) which allows the fungus to invade the reed stem. Larvae later help the fungus to reach the different layers of vascular bundles and finally the medulla. The fungus is apparently unable to join the medulla without the larvae and can not progress up the stem (fig. 4).

Galls of *L. arundinis* on the stems of reed appear to be ambrosia galls. As in the case of ambrosia beetles, the gall midge collects and carries its symbiont in specialized structures: the mycangia. This is the first reported observation of fungal collecting and transporting behaviour by gall midges and also the first description of mycangial pouches in the genus *Lasioptera*. These fungus collecting structures are localized on the cercus. They are composed of two lateral pouches, associated with spoon-like setae. These large hooked setae have also been observed on *L. hungarica* (Skuhravá & Skuhravý 1981) and *L. rubi* (Tastás-Duque & Sylvén 1988) where they were thought to serve as sensilla which register chemical stimuli both by olfaction and contact. My observations have shown that the sensilla are able to discriminate the fungal conidia not only by olfaction but also by their shape and size and thus they may help the midge to find its symbiont on the leaf sheath of the right internode. It is possible that chemical signals are involved in recognizing the correct fungal symbiont. Thus it is incorrect to conclude that the ovipositor picks up fungal conidia similar to the way in which a finger picks up objects when it is run across a surface.

The gall of *L. arundinis* is therefore a model of mu-

tualistic association between a gall midge and its fungal symbiont where both organisms feed and develop on the stems of reed. Together they are able to enter into the stem tissues and feed on vascular tissues with the midge larvae also feeding on the fungus; they first feed on young, tender hyphae budding in the larval chamber and later on the older mycelium (Rohfritsch 1992). The larvae do not only use the mycelium to enter the reed stem and locate food, but to attain protection as well. In this association, the fungus is dispersed into a highly specialized and protected niche. With the help of gall-midge larvae, it gains access to young tissues of the elongating shoot.

It also is apparent that coevolution between gall midges and fungi has taken place as evidenced by physical traits such as the mycangia of the imago and the long bristles and spines of the larvae. There are also behavioural traits of the insect for collecting and carrying specific fungal spores and to inoculate them in a specific manner in the plant tissue along a line which is the larval dispersal route. Success of this plant–fungus–larva interaction is mainly dependent on larval behavioral traits. The fungus is controlled by plant defense reactions and by the larvae and, the insect appears to control physiological processes and morphogenetic expression of the fungus including conidia germination, stimulation of mycelial growth and control over this growth.

Ambrosia gall midges have evidently evolved the means of using and manipulating the fungus, and the fungus may have reciprocated by evolving similarly accommodating traits such as fructifications on upper internodes of attacked shoots, along with a particular size and structure of conidia. The fungus in turn shifted from a saprophytic to a biotrophic mode of life and has avoided plant defense reactions. The fungus now remains under the control of the gall midge to avoid competition for food and space and the proliferating dense mycelium never destroys the overwintering larvae.

Thus ambrosia galls such as this result from the activity of both the insect and the fungus. Cell wall maceration is produced by the fungus but cell activation to high proteosynthesis which characterize nutritive tissues of most gall-inducing insects can also be induced by biotrophic fungus. Furthermore, the hypertrophic gall growth may result from the activity of both organisms. The fungus does not reach the medulla in the upper internodes where it develops without the larva; it does not develop a thick mycelium and has less influence on growth of the internode. Consequently, both organisms stay in a narrow mutualistic relationship. The fungus is collected, transported and deposited via highly evolved mycangia. According to Bissett & Borkent (1988), all *Lasioptera* carry the same kind of conidia, all from the genus *Macrophoma*. It thus can be speculated that all *Lasioptera* female imagoes have mycangia beneath the spoon-like spines on their cerci. To observe the conidia in the mycangia, it is necessary to give the midge the opportunity to collect them and it is essential to avoid ethanol for material preservation or observation.

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